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▶ **To cite this version:**

Marc Le Vée, Astrid Bacle, Elodie Jouan, Valérie Lecureur, Sophie Potin, et al.. Induction of multidrug resistance-associated protein 3 expression by diesel exhaust particle extract in human bronchial epithelial BEAS-2B cells. Toxicology in Vitro, 2019, 58, pp.60-68. 10.1016/j.tiv.2019.03.021 . hal-02091216

HAL Id: hal-02091216

https://univ-rennes.hal.science/hal-02091216

Submitted on 11 Apr 2019

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Induction of multidrug resistance-associated protein 3 expression by diesel exhaust particle extract in human bronchial epithelial BEAS-2B cells

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Abstract

Diesel exhaust particles (DEPs) are common environmental air pollutants known to impair expression and activity of drug detoxifying proteins, including hepatic ATP-binding cassette

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(ABC) drug transporters. The present study was designed to determine whether organic DEP extract (DEPe) may also target ABC drug transporters in bronchial cells. DEPe (10 µg/mL) was demonstrated to induce mRNA and protein expression of the multidrug resistance-associated protein (MRP) 3 in cultured bronchial epithelial BEAS-2B cells, whereas mRNA levels of other MRPs, multidrug resistance gene 1 or breast cancer resistance protein were unchanged, reduced or not detected. DEPe also increased MRP3 mRNA expression in normal human bronchial epithelial cells. Inhibition of the aryl hydrocarbon receptor (AhR) pathway by AhR antagonists or AhR silencing, as well as the silencing of nuclear-factor-E2-related factor 2 (Nrf2) repressed DEPe-mediated MRP3 induction. This underlines the implication of the AhR and Nrf2 signaling cascades in DEPe-mediated MRP3 regulation. DEPe was additionally demonstrated to directly inhibit MRP activity in BEAS-2B cells, in a concentration-dependent manner. Taken together, these data indicate that DEPs may impair expression and activity of MRPs, notably MRP3, in human bronchial cells, which may have consequences in terms of lung barrier and toxicity for humans exposed to diesel pollution.

Key-words: Drug transporter; MRP3; diesel exhaust; bronchial cells; aryl hydrocarbon receptor; Nrf2.

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1. Introduction

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Human ATP-binding cassette (ABC) drug transporters are plasma membrane proteins mediating cellular efflux of xenobiotics (Schinkel and Jonker, 2003). They have been historically characterized as efflux pumps reducing anticancer drug accumulation in cancer cells and thus conferring multidrug resistance (MDR) (Glavinas et al., 2004). They have next been shown to be expressed at various anatomical/histological sites, known to play a major role for xenobiotic disposition, such as intestine, blood-tissue barriers like the blood-brain barrier, liver and kidney (Konig et al., 2013). In this way, ABC drug transporters play a major role in the different steps of pharmacokinetics, which are absorption, distribution and hepatic and renal elimination (Giacomini et al., 2010), and beyond, in drug efficacy and toxicity (DeGorter et al., 2012). Modulation of their activity and/or their expression may therefore have notable consequences for drug disposition and activity, and has consequently to be addressed during the pharmaceutical development of new molecular entities according to a regulatory point of view (Prueksaritanont et al., 2013).

ABC transporters, including P-glycoprotein, encoded by the *MDR1* (*ABCB1*) gene, breast cancer resistance protein (BCRP/*ABCG*2) and multidrug resistance-associated protein (MRP) 1 (*ABCC1*), are also expressed in the lung epithelium (Courcot et al., 2012; Endter et al., 2007; Sakamoto et al., 2015). They therefore likely contribute to the lung barrier and, in this way, may be involved in pulmonary absorption of xenobiotics (Bosquillon, 2010; Ehrhardt et al., 2017), including that of noxious compounds such as air pollutants and cigarette smoke components (van der Deen et al., 2005). They may also participate in lung protection towards volatile toxicants (Leslie et al., 2005). They may additionally be involved in the release of inflammatory mediators such as uric acid, leukotrienes and prostaglandins, which are substrates for some ABC transporters (Nakayama et al., 2011; Rius et al., 2008). This may contribute to lung toxicity caused by air pollutants (Gold et al., 2016; Schneider et al., 2005). The potential relationship between ABC transporters and atmospheric pollutants is

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moreover supported by the fact that diesel exhaust particle (DEP) extract (DEPe) as well as cigarette smoke condensate (CSC) have been shown to induce expression of BCRP, MRP3 (*ABCC3*) and MRP4 (*ABCC4*) in cultured human hepatic cells (Le Vee et al., 2015; Sayyed et al., 2016). However, whether pulmonary expression of ABC drug transporters may also be regulated by inhaled pollutants remains mainly unknown. The lung being the primary and main site of exposure to these pollutants, their effects on pulmonary expression of ABC drug transporters are probably important to assess. In order to gain insights about this point in the present study, and owing to the relevance of human exposure to diesel exhaust (Steiner et al., 2016), we have analyzed the effects of DEPe on ABC drug transporter expression in human bronchial epithelial BEAS-2B cells, used here as *in vitro* lung epithelium model responsive to DEPe (Cao et al., 2010; Le Vee et al., 2016). Our data demonstrate that treatment by DEPe induces expression of the ABC transporter MRP3 in BEAS-2B cells, in an aryl hydrocarbon receptor (AhR)- and nuclear-factor-E2-related factor 2 (Nrf2)-dependent manner. Such data support the idea that air pollutants, including DEPs, may target ABC transporters in the lung.

2. Materials and Methods

2.1 Chemicals

DEPe used in the study was the standard reference material (SRM) 1975, provided by the National Institute of Standards and Technology (Gaithersburg, MD, USA). It is a dichloromethane extract of filter-collected combustion particulate matter, referenced as SRM 2975, from operating forklifts with diesel engines (Hughes et al., 1997). It notably contains polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs (Manzano et al., 2013). For cell exposure, dichloromethane was evaporated under nitrogen and the final residue was dissolved in dimethyl sulfoxide (DMSO). Probenecid, sulforaphane, benzo(a)pyrene (B(a)P),

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StemRegenin-1 and pifithrin-α were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), whereas 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories (Cambridge, MA, USA). Carboxy-2,7-dichlorofluorescein (CF) diacetate was from Life Technologies (Paisley, United Kingdom).

2.2 Cell culture

The human bronchial epithelial cell line BEAS-2B as well as normal human bronchial epithelial (NHBE) cells (isolated from epithelial lining of airways above bifurcation of the lungs from normal donors and provided by Lonza, Basel, Switzerland) were cultured in BEBM^{TM} bronchial epithelial cell growth basal medium, supplemented with BEGM^{TM} bronchial epithelial cell growth medium BulletKit^{TM} (Lonza). BEAS-2B cells were usually seeded at 18 x 10^3 cells/cm^2 and treated by DEPe at confluency stage. Chemicals were initially dissolved in DMSO and stored frozen as stock solutions until use. Final concentration of solvent in culture media did not exceed 0.2% (vol/vol); control cultures received the same dose of DMSO as for their treated counterparts. When chemical inhibitors were used, they were added 15 min before DEPe treatment.

2.3 Cytotoxicity assay

Cellular apoptosis or necrosis were investigated through cell staining with 10 µg/mL Hoechst 33342 and 1 µg/mL propidium iodide for 15 min at 37 °C, as previously described (Sayyed et al., 2016). Apoptotic cells, i.e., cells with condensed blue chromatin or fragmented blue nuclei, and necrotic cells, i.e., cells with red nuclei, were next counted in comparison with total cell population using fluorescence microscopy. The percentage of viable cells, defined as non-apoptotic and non-necrotic cells, was deduced by subtracting the percentages of apoptotic and necrotic cells from 100% (total cell number).

2.4 RNA isolation and analysis

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Total RNAs were extracted from cells using the TRI reagent (Sigma-Aldrich), and were then reverse-transcribed to cDNA using the reverse-transcription (RT) kit from Applied Biosystems (Foster City, CA, USA). Quantitative polymerase chain reaction (qPCR) assays were next performed using the fluorescent dye SYBR Green methodology and a CFX384 real-time PCR detection system (Bio-Rad, Marnes-la-Coquette, France), as previously described (Mayati et al., 2018). Gene primers are described in Table S1. The specificity of each gene amplification was verified at the end of qPCR reactions through analysis of dissociation curves of the PCR products. Amplification curves were analyzed with CFX Manager software (Bio-Rad), using the comparative cycle threshold method. Relative quantification of the steady-state target mRNA levels was calculated after normalization of the total amount of cDNA tested to the 18S rRNA endogenous reference, using the $2^{(-\Delta\Delta Ct)}$ method. Data were finally commonly expressed as fold change comparatively to control untreated cells or in arbitrary units relatively to 18S rRNA content (Moreau et al., 2011).

2.4 RNA interference assays

Control non-targeting siRNAs (siNT) or siRNAs targeting AhR (siAhR) or Nrf2 (siNrf2), provided by Sigma-Aldrich and prepared in Opti-MEM medium (ThermoFischer Scientific, Waltham, MA, USA) at a final 0.4 µM concentration, were incubated overnight at 37°C in the presence of 12 µL/mL of Lipofectamine RNAiMax ™ (ThermoFisher Scientific) with BEAS-2B cells plated in 24 wells plates. Transfected BEAS-2B cells were next maintained in usual medium for 48 h before treatment by DEPe.

2.5 Western-blotting

Total protein extracts were prepared from BEAS-2B cells as previously reported (Le Vee et al., 2016). Protein were then separated on polyacrylamide gel and electrophoretically transferred onto Protan® nitrocellulose membranes (Whatman GmbH, Dassel, Germany). After blocking with Tris-buffered saline containing 4 % (vol/vol) bovine serum albumin and

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0.1% (vol/vol) Tween 20 for 30 min at room temperature, membranes were incubated overnight at 4°C with primary antibodies against MRP3 (clone M3II-9) (Enzo Life Sciences, Villeurbanne, France) or p38 (clone C20) (Santa Cruz Biotechnology, Dallas, TX, USA). After washing, membranes were next re-incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark). Immunolabeled proteins were finally visualized by chemiluminescence. Gel loading and transfer were verified by staining membranes with Ponceau red. Densitometry with ImageJ 1.40g software (National Institutes of Health, Bethesda, MD, USA) was used to quantify intensities of stained bands and for normalization to p38 content.

2.6 MRP activity assay

MRP activity was analyzed through measuring intracellular retention of the MRP substrate CF, as previously described (Jouan et al., 2016). Briefly, BEAS-2B cells were incubated at 37°C with 3 µM CF diacetate for 30 min. After washing in phosphate-buffered saline, cells were re-incubated in CF diacetate-free medium at 37°C for 60 min in the presence or absence of DEPe or of 2 mM probenecid, a reference inhibitor of MRPs (Rosati et al., 2004). Intracellular retention of CF was next determined by spectrofluorimetry (excitation and emission wavelengths were 485 and 535 nm, respectively) using a SpectraMax Gemini SX spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA). Protein content was in parallel determined by the Bradford method. Data were expressed as % of CF initial loading, as CF fluorescence arbitrary unit/mg protein or as % of MRP activity according to the following equation:

$$\%\ \text{MRP activity} = \frac{(\text{CF Retention}^{\text{Probenecid}} - \text{CF Retention}^{\text{DEPe}}) \times 100}{\text{CF Retention}^{\text{Probenecid}} - \text{CF Retention}^{\text{Control}}} \qquad (1)$$

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with CF Retention^{Probenecid} = CF retention in the presence of probenecid, CF Retention^{DEPe} = CF retention in the presence of DEPe and CF Retention^{Control} = CF retention in control cells not exposed to probenecid or DEPe.

2.7 Statistical analysis

Quantitative data were usually expressed as means ± SEM. Data were statistically analyzed using Student's *t* test or analysis of variance (ANOVA) followed by Dunnett's or Newman-Keuls post-hoc test. The criterion of significance was $p < 0.05$. Half maximal inhibitory concentration (IC_{50}) value of DEPe towards MRP activity was determined using GraphPad Prism software (GraphPad Software, La Jolla, CA), through nonlinear regression based on the four parameter logistic function.

3. Results

3.1 Induction of MRP3 expression by DEPe

BEAS-2B cells were first exposed for 48 h to 10 µg/mL DEPe, a DEPe concentration in the range of those previously used for treating cultured cells (Bach et al., 2015; Le Vee et al., 2015; Mundandhara et al., 2006). Such a treatment failed to trigger apoptosis or necrosis (Fig. 1). Among ABC transporter mRNA expressions, that of *MDR1* was not detected in both untreated control BEAS-2B cells and DEPe-exposed counterparts (Fig. 2A). Those of BCRP, MRP2, MRP5 and MRP6 were low in untreated BEAS-2B cells (mRNA level means < 2 arbitrary units) and were either unchanged (BCRP and MRP5) or reduced (MRP2 and MRP6) by DEPe (Fig. 2A). MRP1, MRP3 and MRP4 were the most expressed ABC drug transporters in BEAS-2B cells (mRNA level means > 8 arbitrary units). DEPe treatment decreased MRP1 and MRP4 levels, by 1.5 ± 0.2-fold and 1.6 ± 0.1-fold factors, respectively; by contrast, it induced that of MRP3 by a 3.3 ± 0.9-fold factor. As (i) MRP3 was the ABC drug transporter exhibiting the higher mRNA expression in BEAS-2B cells (Fig. 2A), (ii) it was the only to

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display mRNA up-regulation in response to DEPe and (iii) it may be implicated in lung diseases, notably in lung cancers (Hanada et al., 2008; Young et al., 1999), we decided to focus the next studies on DEPe-mediated MRP3 up-regulation. Time-course analysis (Fig. 2B) indicated that a short DEPe-treatment (8 h) failed to induce MRP3 mRNA expression, in contrast to longer treatments (24 h and 48 h). DEPe-mediated MRP3 mRNA up-regulation was next shown to be concentration-dependent, i.e., it required a 10 µg/mL DEPe concentration, whereas lower concentrations (from 0.1 to 5 µg/mL) were inactive (Fig. 2C). The effects of DEPe concentrations higher than 10 µg/mL on MRP3 mRNA levels were not studied as such concentrations were found to be cytotoxic (data not shown). DEPe was demonstrated to enhance MRP3 protein expression in BEAS-2B cells by a 2.6-fold factor (Fig. 2D). Finally, we investigated the effects of DEPe on MRP3 expression in NHBE cells from three donors. Constitutive MRP3 mRNA levels were clearly detectable (mRNA level>2 arbitrary units) in NHBE cells from each donor (Fig. S1). DEPe (10 µg/ mL) was found to induce MRP3 mRNA expression in these NHBE cells by a 5.7 ± 0.7-fold factor (Fig. 2E).

3.2 Inhibition of MRP activity by DEPe

DEPe has previously been shown to inhibit MRP2-like activity in human hepatic HepaRG cells (Le Vee et al., 2015). As MRPs, notably MRP1, MRP2 and MRP3, share numerous inhibitors (Payen et al., 2000; Zhou et al., 2008), DEPe may be postulated to also block MRP activity in bronchial BEAS-2B cells. To test this hypothesis, we initially loaded BEAS-2B cells with the generic MRP substrate CF and next analyzed the efflux of the dye in the absence or presence of 10 µg/mL DEPe or of 2 mM probenecid. As shown in Fig. 3A, BEAS-2B cells exhibited probenecid-inhibitable efflux of CF, indicating that they constitutively displayed MRP activity. Addition of 10 µg/mL DEPe during the efflux period significantly enhanced CF retention in BEAS-2B cells, indicating that DEPe inhibited MRP activity. This inhibitory effect towards MRP activity was next shown to be concentration-

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dependent (DEPe IC_{50} value = 10.0 µg/mL) (Fig. 3B). We next investigated whether BEAS-2B cells exposed to DEPe treatment conditions known to trigger MRP3 induction, i.e., a 48 h exposure to 10 µg/mL DEPe, exhibit impaired MRP activity. For such a purpose, DEPe was withdrawn during the efflux assay, in order to study only the effects of the prior 48 h exposure to DEPe. As indicated in Fig. 3C, the levels of CF retention after the efflux phase were not different in both untreated and DEPe-exposed cells.

3.3 Implication of AhR in DEPe-mediated MRP3 induction

The regulatory effects of DEPe towards hepatic transporter expression implicate AhR (Le Vee et al., 2015), a xenobiotic-sensing receptor known to be activated by some components of DEPe, notably PAHs (Mason, 1994; Palkova et al., 2015). We therefore studied the implication of AhR in DEPe-mediated induction of MRP3 in bronchial BEAS-2B cells. The AhR pathway was first shown to be activated by DEPe in BEAS-2B cells, as demonstrated by (i) the up-regulation of the reference AhR target gene cytochrome P-450 (CYP) 1B1 (Nebert et al., 2004) in DEPe-exposed BEAS-2B cells and (ii) the suppression of this up-regulation by co-treatment with StemRegenin-1, a potent antagonist of AhR (Boitano et al., 2010) (Fig. 4A). CYP1B1 mRNA induction was maximal at an 8 h exposure to DEPe (Fig. S2) and was thus faster than that of MRP3 (Fig. 2B). The AhR blocker StemRegenin-1 hindered MRP3 mRNA up-regulation due to DEPe (Fig. 4A). Transfection of BEAS-2B cells with siRNAs directed against AhR, resulting in a strong repression of AhR mRNA by a 3.8 ± 0.3-fold factor (Fig. S3), inhibited DEPe-mediated up-regulation of CYP1B1 and MRP3 (Fig. 4B). To determine if AhR activation was sufficient to trigger MRP3 induction, we next studied the effects of TCDD, a potent and reference activator of AhR (Okey et al., 1994). As shown in Fig. 4C, TCDD markedly induced mRNA expression of CYP1B1, but failed to enhance that of MRP3. By contrast, exposure of BEAS-2B cells to the prototypical PAH B(a)P increased both CYP1B1 and MRP3 mRNA levels (Fig. 4C).

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3.4 Implication of Nrf2 in DEPe-mediated MRP3 induction

As Nrf2, a transcription factor activated by oxidative stress (Nguyen et al., 2004), is involved in both MRP3 regulation (Mahaffey et al., 2009) and DEP effects (Jaguin et al., 2015; Li et al., 2004), we analyzed its role in MRP3 up-regulation by DEPe. We first demonstrated that the Nrf2-related MRP3 regulation pathway was active in BEAS-2B cells, through showing that sulforaphane, a potent Nrf2 activator (Thimmulappa et al., 2002), induced MRP3 mRNA and protein expression in BEAS-2B cells (Fig. 5A and 5B). DEPe exposure was next shown to induce mRNA expression of the reference Nrf2 target genes NQO1 (Tanigawa et al., 2007) and HO-1 (Alam and Cook, 2003) (Fig. 5C), indicating that the Nrf2 signaling pathway was activated by DEPe in BEAS-2B cells, as already demonstrated in other lung cell lines (Baulig et al., 2003). Transfection of BEAS-2B cells by siRNAs targeting Nrf2, which reduced Nrf2 mRNA expression by a 3.7 ± 0.4-fold factor (Fig. S3), markedly decreased MRP3 mRNA induction by DEPe and sulforaphane (Fig. 5D). By contrast, Nrf2 silencing did not impair up-regulation of CYP1B1 mRNA levels by DEPe. Sulforaphane failed to increase CYP1B1 expression (Fig. 5D) and its inducing effect towards MRP3 and NQO1 mRNA levels was not impaired by AhR silencing (Fig. S4). Besides, AhR or Nrf2 down-regulations by siRNA transfection repressed NQO1 mRNA induction by DEPe (Fig. S5). Finally, pifithrin-α, a potent inhibitor of CYP1A1/1B1 activity preventing the formation of PAH metabolites (Sparfel et al., 2006), failed to inhibit MRP3 up-regulation due to DEPe (Fig. S6).

4. Discussion

The present study demonstrates that DEPe is able to induce MRP3 expression in bronchial epithelial cells. Indeed DEPe increased MRP3 mRNA levels in both BEAS-2B cells

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and NHBE cells. It concomitantly induced MRP3 protein expression in BEAS-2B cells. This up-regulation of MRP3 by DEPe was concentration-dependent, i.e., it requires the use of 10 µg/mL DEPe. Such a concentration failed to trigger cytotoxicity in BEAS-2B cells. MRP3 up-regulation was consequently not the consequence of an unspecific toxicity caused by DEPe. The specificity of the MRP3 up-regulation by DEPe is additionally supported by the fact that DEPe did not induce expression of other ABC transporters; indeed, MRP1 and MRP4 mRNA expressions were rather reduced by DEPe, whereas those of MRP5 and BCRP were unchanged. With respect to *MDR1*, we failed to detect its expression at mRNA levels, in both untreated and DEPe-treated BEAS-2B cells. Drug transporter quantification by liquid chromatography-tandem mass spectrometry also failed to detect P-glycoprotein in BEAS-2B cells (Sakamoto et al., 2015). Taken together, these data suggest that BEAS-2B cells do not constitutively express P-glycoprotein/*MDR1* and are thus probably not a valuable model for studying P-glycoprotein/*MDR1* regulation in bronchial cells. Additionally, BEAS-2B cells unfortunately failed to polarize and form tight junctions, and exhibited only very low, if any, transepithelial electrical resistance (TEER) values (Stewart et al., 2012). This precludes their use for *in vitro* characterization of the effects of air pollutants like DEP on vectorial transport across the lung barrier.

The mechanisms responsible for MRP3 induction by DEPe in BEAS-2B cells likely implicate AhR, a xenobiotic-sensing transcription factor already involved in DEPe-mediated regulation of hepatic drug transporters (Le Vee et al., 2015). Indeed, the AhR antagonist StemRegenin-1, as well as AhR silencing through siRNA transfection, repressed MRP3 induction in DEPe-exposed BEAS-2B cells. It is however noteworthy that the potent AhR ligand TCDD failed to increase MRP3 mRNA levels in BEAS-2B cells, thus indicating that AhR activation is not sufficient to induce MRP3 expression. Factors additional to AhR are consequently required for MRP3 up-regulation and, among these putative factors, Nrf2 has to

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be considered with priority. This transcription factor is primarily responsive to oxidative or electrophilic stress, which prevents its degradation by the proteasome and subsequently triggers its translocation from the cytoplasm into the nucleus, where it binds to antioxidant response element (ARE) found in the upstream promoter of target genes, and initiates their transcription (Nguyen et al., 2004). Nrf2 is well known to be activated by DEPs (Li et al., 2004), as illustrated by the up-regulation of NQO1 and HO-1, two reference Nrf2-responsive genes, in DEPe-exposed BEAS-2B cells. Its implication in DEPe-mediated induction of MRP3 is supported by the following points: (i) treatment by the reference Nrf2 activator sulforaphane triggered MRP3 induction in BEAS-2B cells, (ii) suppression of Nrf2 expression by siRNA transfection repressed MRP3 up-regulation due to DEPe, (iii) analysis of the MRP3 promoter sequence has revealed the presence of multiple AREs (Mahaffey et al., 2009), mediating direct regulation of MRP3 by Nrf2 (Mahaffey et al., 2012) and (iv) Nrf2, in association with AhR, has already been demonstrated to be involved in various phenotypic effects of DEPe, notably in human macrophages (Jaguin et al., 2015).

The exact way by which AhR and Nrf2 cooperate to induce MRP3 expression in response to DEPe remains to be determined. Basic implication of the AhR pathway in that of Nrf2 or, inversely, basic involvement of the Nrf2 signaling cascade in that of AhR is very unlikely. Indeed, silencing of Nrf2 is unable to hinder AhR-related induction of the AhR reference target CYP1B1 by DEPe, whereas that of AhR failed to impair up-regulation of the Nrf2 target NQO1 by sulforaphane. Additionally, the two ways are probably not involved at the same level in MRP3 up-regulation. Indeed, if basic activation of the Nrf2 signaling pathway by the reference activator sulforaphane is sufficient to induce MRP3 expression, likely via the AREs found in the upstream promoter region of the MRP3 gene (Mahaffey et al., 2009), it is not the case for the basic stimulation of the AhR signaling cascade by the reference AhR agonist TCDD. In this context, it is noteworthy that the AhR way is well-

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known to play a major role in the metabolism of some of these ligands, notably PAHs, through inducing the expression of PAHs-metabolizing enzymes like CYP1A1 and CYP1B1 (Nebert et al., 2004). Some of these PAH metabolites are known to be electrophilic and to induce oxidative stress (Nebert et al., 2000). They may thus contribute in a major way to the activation of the Nrf2 transcription battery by PAHs (Souza et al., 2016). It is therefore tempting to speculate that the MRP3 induction by DEPe, as well as that of NQO1, which also depends on AhR and Nrf2 (Fig. S5), may require, first, the AhR-dependent formation of electrophilic/pro-oxidative metabolites of some DEPe chemical components, notably PAHs, and, secondly, the activation of the Nrf2 pathway by these metabolites or by the oxidative stress associated with the generation of these metabolites. This may finally result in increased transcription of the MRP3 gene via AREs. This hypothesis is supported by the fact that the PAH B(a)P, which is a component of diesel exhaust and which generates electrophilic metabolites, in contrast to TCDD (van Grevenynghe et al., 2004), induced MRP3 expression in BEAS-2B cells. Moreover, the time-course of MRP3 mRNA induction by DEPe indicates a rather delayed response, i.e., a shorter exposure (8 h) was ineffective, which may also argue in favor of the requirement of metabolite formation. By contrast, the pure AhR target CYP1B1 was induced after an 8 h exposure to DEPe. CYP1B1, as well as CYP1A1, well known to generate electrophilic PAH metabolites (Nebert et al., 2004), are however unlikely to be involved in MRP3 up-regulation in DEPe-exposed BEAS-2B cells. Indeed, blockage of their activity using the CYP1A1/1B1 inhibitor pifithrin-α failed to inhibit MRP3 induction by DEPe.

Besides increasing MRP3 expression, DEPe directly inhibited MRP activity in BEAS-2B cells, in a concentration-dependent manner (Fig. 3A and 3B). This agrees with the inhibitory effects of DEPe towards MRP2-like activity in human hepatic cells (Le Vee et al., 2015). Such data favor the idea that some chemical components of DEPe may act as potent

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inhibitors of MRP activity. However, these DEPe chemicals remain to be precisely identified. They may also be present in other pulmonary pollutants, such as the cigarette smoke, which hinders MRP1 activity in lung epithelial cells (van der Deen et al., 2007). The direct inhibition of MRP activity by DEPs and the regulation of MRP expression, notably of MRP3, by DEPe, may be difficult to interpret in terms of the final effects of DEPe towards MRPs. It is however noteworthy that the MRP activity inhibition by DEPe was rather reversible, i.e., pre-treatment by DEPe for 48 h following by its withdrawal during the MRP activity test, failed to inhibit MRP activity (Fig. 3C), whereas such a 48 h treatment by DEPs induced MRP3 expression (Fig. 2B and Fig. 2D). This suggests that the regulation of MRP expression, notably MRP3 induction, may correspond to a more sustainable consequence to DEP exposure than the acute inhibition of MRP activity. In this context, it is noteworthy that MRP3 induction after a 48 h exposure to DEPe did not result in decreased CF retention, suggesting a lack of stimulatory effect towards MRP activity. Such data may be due to the fact that the probe used for the MRP activity assay, i.e., CF, is shared by various MRPs, as most of MRP3 substrates (Payen et al., 2000; Zhou et al., 2008); therefore, the CF-based efflux assay likely reflects global MRP activity, not strictly that of MRP3. Nevertheless, as MRP3 has been hypothesized to be a cellular eliminator of toxins from both endogenous and exogenous sources in lung cells (Torky et al., 2005), its increased expression in response to DEPe may serve as an adaptive and protective response to the potential toxicity of DEP components. MRP3 induction may notably facilitate anti-oxidant activities within DEP-exposed bronchial cells, as recently proposed for MRP3 up-regulation in airway epithelial cells from smokers (Aguiar et al., 2019).

Whether the MRP3 regulation occurring in DEPe-treated cultured bronchial cells may be relevant in humans environmentally exposed to DEPs is probably a key-point to determine. This likely requires to consider the concordance between *in vitro-in vivo* dosimetry, which

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represents a common challenge in air pollutant toxicology (Donaldson et al., 2008; Klein et al., 2017; Oberdorster and Finkelstein, 2006). The mean DEP exposure in the United States of America is estimated to be 2 $\mu g/m^3$, but concentrations in vehicles and by major streets and highways can approach 25 $\mu g/m^3$, with "hot spots" of ambient exposure reaching around 100-200 $\mu g/m^3$ (Ghio et al., 2012); some occupational activities, notably in mines, result in much greater levels of exposure (up to 653 $\mu g/m^3$) (Pronk et al., 2009). Based on *in vivo* calculations for high-risk individuals exposed to particulate matter levels of 79 $\mu g/m^3$, deposition rates of fine particulate matter (PM 2.5), which represents an important fraction of DEP (Wichmann, 2007), have been estimated over 24 h to be 2.3 $\mu g/cm^2$ in the tracheobronchial and 0.05 $\mu g/cm^2$ in the alveolar regions (Li et al., 2003). Importantly, the 10 µg/mL DEPe concentration inducing MRP3 expression in BEAS-2B cells corresponds to approximately 2 $\mu g/cm^2$ equivalent DEP dose according to previous conversions of *in vitro* DEPe/DEP dose to DEP dose/unit surface area (Li et al., 2003). MRP3 expression may therefore be induced in tracheobronchial, but not in alveolar areas, of humans highly exposed to DEP. The fact that MRP3 mRNA expression has been recently shown to be induced in airway epithelial cells from smokers when compared to counterparts from non-smokers (Aguiar et al., 2019) additionally supports the conclusion that MRP3 may be a relevant target for air pollutants like DEP and cigarette smoke in the human lung.

In summary, exposure of bronchial epithelial BEAS-2B cells to DEPe resulted in AhR- and Nrf2-related induction of the ABC drug transporter MRP3. DEPe additionally directly inhibited MRP activity in BEAS-2B cells. Such data suggest that membrane transporters, including ABC transporters, but also solute carrier (SLC) transporters like the heterodimeric amino acid transporter LAT1 (*SLC7A5*)/CD98hc (*SLC3A2*) already demonstrated to be regulated by DEPe in BEAS-2B cells (Le Vee et al., 2016), are targets for DEPs in bronchial epithelial cells. Further studies are likely required to precise the potential

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relevance of such regulations in humans exposed to air pollution, notably in terms of lung barrier and lung toxicity.

Acknowledgments

The authors thank Mrs M. Febvre-James for language editing of the manuscript.

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Legends to figures

Figure 1. Effects of DEPe exposure on BEAS-2B cell viability. BEAS-2B cells were either untreated (CTR) or exposed to 10 µg/mL DEP for 48 h. Apoptotic and necrotic cells were next numerated as described in Materials and Methods. Data are expressed as percentages of total number of cells and are the means ± SEM of three independent assays.

Figure 2. Induction of MRP3 expression in response to DEPe. (A) BEAS-2B cells were either untreated (CTR) or exposed to 10 µg/mL DEPe for 48 h. ABC drug transporter mRNA expression was then analyzed by RT-qPCR. Data are expressed as arbitrary units and are the means ± SEM of five independent assays. *, $p < 0.05$ when compared to control untreated cells. (B, C) BEAS-2B cells were either untreated (CTR) or exposed to (B) 10 µg/mL DEPe for various lengths of time (from 8 h to 48 h) or (C) various DEPe concentrations (from 0.01 to 10 µg/mL) for 48 h. MRP3 mRNA expression was next determined by RT-qPCR. Data are expressed as fold change comparatively to MRP3 mRNA levels found in untreated control cells and are the means ± SEM of (B) five or (C) three independent assays. *, $p < 0.05$ when compared to control untreated cells. (D) BEAS-2B cells were either untreated (CTR) or exposed to 10 µg/mL DEPe for 48 h. MRP3 protein expression was next determined by Western-blotting. A representative blot is shown in (D, Left). The results of densitometric analysis of blots and normalization to p38 content from three independent assays, expressed

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as fold change comparatively to MRP3 protein expression in control cells, are indicated in (D, Right). *, $p < 0.05$ when compared to control cells. (E) NHBE cells were either untreated (CTR) or exposed to 10 µg/mL DEPe for 24 h. MRP3 mRNA expression was then analyzed by RT-qPCR. Data are expressed as fold change comparatively to MRP3 mRNA levels found in untreated control cells and are the means ± SEM of values from three independent NHBE cell populations. *, $p < 0.05$ when compared to control untreated cells.

Fig. 3. Effects of DEPe towards MRP activity. (A) BEAS-2B cells, initially loaded with the MRP substrate CF used under its diacetate ester form, were re-incubated in CF-free medium in the absence (CTR) or presence of 2 mM probenecid or 10 µg/mL DEPe for 60 min. Intracellular retention of CF was then determined by spectrofluorimetry. Data are expressed as % of initial CF loading and are the means ± SEM of three independent assays. *, $p < 0.05$ when compared to control cells. (B) CF-loaded BEAS-2B cells were re-incubated in CF-free medium in the absence or presence of 2 mM probenecid or various concentrations of DEPe. MRP activity was then determined as described in Materials and Methods. Data are expressed as % of MRP activity found in control cells and are the means ± SEM of three independent assays. DEPe IC_{50} value is indicated at the top of the graph. (C) BEAS-2B cells were either untreated (CTR) or exposed to 10 µg/mL DEPe for 48 h. After washing, cells were loaded with CF used under its diacetate form for 30 min and then re-incubated in CF-free medium for 60 min. Intracellular levels of CF were finally determined by spectrofluorimetry. Cellular concentrations of CF are expressed in fluorescence arbitrary units (FAU)/mg protein and are the means ± SEM of four independent assays. *, $p < 0.05$; NS, not statistically significant.

Fig. 4. Implication of AhR in DEPe-mediated MRP3 induction. (A) BEAS-2B cells were either untreated (CTR) or exposed to 10 µg/mL DEPe, 5 µM StemRegenin-1 (SR) or DEPe/SR for 48 h. (B) BEAS-2B cells transfected with non-targeting siRNAs (siNT) or with siRNAs directed against AhR (siAhR) were either untreated (CTR) or exposed to 10 µg/mL

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DEPe for 48 h. (C) BEAS-2B cells were either untreated (CTR) or exposed to 10 nM TCDD or 10 µM B(a)P for 48 h. (A-C) CYP1B1 and MRP3 mRNA expression was next determined by RT-qPCR. Data are expressed as fold change comparatively to gene expression in control cells and are the means ± SEM of four independent assays. *, $p < 0.05$; NS, not statistically significant.

Fig. 5. Implication of Nrf2 in DEPe-mediated MRP3 regulation. (A, B) BEAS-2B cells were either untreated (CTR) or exposed to 10 µM sulforaphane (SULFO) for 48 h. (A) MRP3 mRNA expression was determined by RT-qPCR; data are expressed as fold change comparatively to untreated control cells and are the means ± SEM of four independent assays. (B) MRP3 protein expression was determined by Western blotting. A representative blot is shown in (B, Up). The results of densitometric analysis of blots and normalization to p38 content from three independent assays, expressed as fold change comparatively to MRP3 protein expression in control cells, are indicated in (B, Down). (C) BEAS-2B cells were either untreated (CTR) or exposed to 10 µg/mL DEPe for 48 h. NQO1 and HO-1 mRNA expression was then determined by RT-qPCR. Data are expressed as fold change comparatively to untreated control cells and are the means ± SEM of four independent assays. (A-C) *, $p < 0.05$ when compared to control cells. (D) BEAS-2B cells transfected with non-targeting siRNAs (siNT) or with siRNAs directed against Nrf2 (siNrf2) were either untreated (CTR) or exposed to 10 µg/mL DEPe for 48 h. MRP3 and CYP1B1 mRNA expression was then determined by RT-qPCR. Data are expressed as fold change comparatively to untreated control cells and are the means ± SEM of four independent assays. *, $p < 0.05$. NS, not statistically significant.

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Highlights

- DEPe induced mRNA and protein expression of MRP3 in human bronchial BEAS-2B cells
- The transcription factors AhR and Nrf2 are implicated in DEPe-mediated MRP3 induction
- DEPe concomitantly blocked MRP activity in cultured BEAS-2B cells

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